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1 A Method for Inducing the Sexual Maturation of
2 Lugworms
3

4 The present invention relates to the aquaculture of
5 marine worms and particularly to the control of
6 sexual maturation of marine worms.
7

8 Marine worms are animals in the Class *Polychaeta* of
9 the Phylum *Annelida* or in the Phylum *Sipunculida*.
10 Such worms are the natural foodstuff for fish,
11 crustaceans and other marine organisms, and
12 therefore find utility as bait for anglers and
13 other fishermen. Additionally certain marine worms
14 have been extensively studied and are recognised as
15 being useful for toxicity testing and other
16 scientific purposes. Marine worms also find
17 utility as a dietary item for aquaculture either in
18 fresh or frozen form or incorporated into food
19 products in a variety of formulations.
20

21 However, the natural supply of marine worms is
22 finite and serious concerns have been raised

1 regarding the potential environmental damage caused
2 by unsustainable over harvest. An environmentally
3 acceptable alternative to collecting marine worms
4 from the wild is their aquaculture to provide a
5 sustainable supply. The aquaculture of marine
6 worms provides the additional benefit of known and
7 quantified content of specified biochemical content
8 and the certifiable absence of specific pathogenic
9 organisms providing aquaculture feeds that may be
10 designated as having Specific Pathogen Free status.

11
12 The aquaculture of the polychaete worms
13 *Arenicolidae* (commonly known as "lugworms") has
14 attracted some interest (see Gambi et al., 1994;
15 Olive 1993), especially since bait digging for
16 these animals was considered to be a cause of
17 environmental damage (see Olive, 1993).

18
19 *Arenicola marina* (lugworm) is an iteroparous
20 polychaete, breeding several times per lifetime,
21 but at annual intervals (Clark and Olive, 1973).
22 *A. marina* is a marine deposit feeder (Jumars, 1993;
23 Fauchald and Jumars, 1979) and ingests sand grains
24 or other substrate at the head of the horizontal
25 section of a J-shaped burrow in which the animal
26 resides.

27
28 An attempt to culture *A. cristata* was described by
29 D'Asaro et al., 1976 but did not lead to commercial
30 aquaculture of any species of lugworm using the
31 methods described. A more successful methodology
32 for the aquaculture of deposit feeding marine worms
33 has since been described in our published

1 International Patent Application No. WO-A-
2 03/007701. The methodology described relates to a
3 method of successfully farming the worms or their
4 larvae, such that the body weight of the worms
5 increases. However, the methodology described in
6 WO-A-03/007701 offers no means to control the
7 breeding period of the worms.

8
9 D'Asaro describes a method to induce spawning in
10 the lugworm *Arenicola cristata*, by maintaining the
11 broodstock at temperatures of 18 to 32°C. In the
12 wild, female *Arenicola cristata* worms will produce
13 egg masses at frequent intervals throughout the
14 year and D'Asaro describes using temperatures of
15 16-18°C or above to stimulate the release of up to
16 4 egg masses a month for cultured female worms.

17
18 By contrast, the *Arenicola marina* and *Arenicola*
19 *defodiens* populations spawn annually in a discrete
20 period lasting 4 to 5 days. Simultaneous spawning
21 of the local population of a single species in this
22 way is termed "epidemic spawning". The spawning of
23 discrete populations in neighbouring locations may
24 vary by several days or even weeks, whilst the date
25 of spawning - even at a single location - may vary
26 by as much as 4 to 5 weeks in subsequent years.
27 Since *Arenicola marina* exhibits epidemic spawning
28 it has been postulated that external factors could
29 determine, or at least influence, the date of
30 spawning within a single population.

31

32 A study by Watson et al., 2000 examined various
33 external factors (specifically environmental

1 factors) and assessed their influence on the date
2 of spawning within a Scottish population of
3 *Arenicola marina*. The external factors reviewed
4 were the sea and air temperatures, tidal cycle, air
5 pressure, rainfall and windspeed/direction. The
6 study noted that the population studied always
7 spawned on the spring tides and suggested that
8 spawning correlated with the tidal cycle with a
9 semi-lunar periodicity. It was also suggested that
10 a drop in temperature could operate as a cue to
11 spawning, but Watson et al., 2000 concluded that
12 their data did not indicate any threshold
13 temperature or reduction in temperature necessary
14 to induce spawning.

15

16 In conclusion, it is clear from the literature that
17 the lugworms *Arenicola marina* and *Arenicola*
18 *defodiens* reproduce only during a very short period
19 of the year and that the date of spawning is not
20 easily predictable. In terms of the aquaculture of
21 lugworms such as *Arenicola marina* or *Arenicola*
22 *defodiens* that are normally found in temperate or
23 boreal regions, it would be of great benefit to be
24 able to induce the spawning of the worms in order
25 to maintain the farmed population at the levels
26 required.

27

28 We have now found that the careful manipulation of
29 temperature can induce spawning in both male and
30 female marine worms of *Arenicola marina* and
31 *Arenicola defodiens* such that reproduction can be
32 made to occur at all times of the year and this

1 ability to induce sexual maturation represents a
2 significant advance in aquaculture of these worms.

3
4 The present invention thus provides a method of
5 inducing gamete maturation to the point of
6 competence to fertilise in marine worms of the
7 family Arenicolidae which exhibit epidemic
8 spawning, said method comprising:

9 providing maturing male worms and/or maturing
10 female worms wherein said worms are provided
11 in a housing substrate in sea water at a
12 temperature of 4 to 8°C for a time period of
13 14 to 24 days.

14
15 The term "epidemic spawning" as used herein is as
16 defined in Watson et al., 2000 as the synchronised
17 spawning of a local population of a single species.
18 "Epidemic spawning" is thus distinguished from
19 "mass spawning" which is used to describe the
20 synchronised spawning of population of several
21 species at a given locale (see Babcock et al.,
22 1986).

23
24 In one embodiment the worms are maintained at a
25 temperature of approximately 6°C (eg. 5 to 7°C) for
26 14 to 24 days.

27
28 In one embodiment the worms are maintained at a
29 temperature of 4 to 8°C (for example 5 to 7°C) for
30 at least 18 days and typically 20 to 22 days.

31
32 Reference is made above to the worms being held at
33 a temperature of 4 to 8°C (preferably 5 to 7°C) for

1 a period of 14 to 24 days. The exact time period
2 will depend upon the condition of the worms for
3 spawning as assessed by measuring the diameter of
4 the coelomic oocytes (eggs) for female worms, or in
5 male worms by measuring the percentage of the
6 groups of male sperm cells (platelets) wherein the
7 sperm tails have differentiated (morulae) in
8 samples of coelomic fluid obtained by biopsy. The
9 biopsy may be carried out by inserting a hypodermic
10 needle into the tail region of the body parallel to
11 the long axis of the body in order to avoid
12 possible damage to the blood vessels and vital
13 organs present in the non-tail region of the
14 animal's body.

15

16 In one embodiment, the present invention induces
17 spawning (i.e. gamete release) of the worms.
18 However, we have found that the effect of
19 temperature of 4 to 8°C promotes the maturation of
20 gametes so that the gametes are ready for release
21 in spawning under appropriate hormonal control.
22 These mature gametes could be harvested from the
23 parent worm such that fertilisation can occur in
24 vitro. Gamete release can be achieved by the
25 natural release of a hormone or may, if preferred,
26 be achieved by the injection of a homogenate of the
27 prostomium in sterile filtered seawater at a
28 concentration of 1 prostomium equivalent per worm
29 (for females). In the case of male worms gamete
30 release can be induced by injection of 8, 11, 14-
31 eicosatrienoic acid (usually dissolved in methanol
32 and diluted with seawater) to give a final
33 concentration in the body cavity of approximately 1

1 $\times 10^{-4}$ M. Similar procedures are described in the
2 literature (Bentley et al. 1990 and Bentley et al.
3 1996) to induce gamete release from animals ready
4 to spawn during the natural breeding season.

5
6 The present invention is suitable for maturing
7 female worms and for maturing male worms of the
8 family Arenicolidae. Maturing female worms are
9 defined as female worms observed to possess
10 coelomic eggs having a modal diameter of at least
11 160 microns. Usually the observation is made by
12 coelomic biopsy, a technique routine in the art.
13 Briefly, a coelomic biopsy involves removal of a
14 sample of coelomic fluid by means of a hypodermic
15 syringe (a 25g hypodermic needle is suitable) and
16 examining the sample taken by light microscope.
17 Maturing male worms are defined as male worms
18 observed to possess a ratio of morulae to
19 spermatocytes of 80% or more. Usually this
20 observation is made by examining a small sample of
21 coelomic fluid obtained as described above on a
22 microscope slide using a x10 objective lens and
23 examining approximately 100 groups of male germ
24 cells (spermatocytes in the form of platelets or
25 morulae as mentioned above). Maturing worms are
26 present in samples of worms which have been
27 cultured at a temperature of approximately 16°C (eg
28 14 to 18°C) for a period of 3 to 5 months. These
29 maturing worms can be selected for use in the
30 present invention. We have found that allowing the
31 maturing worms to remain at the culture temperature
32 (of approximately 16°C) results in degeneration of

1 the maturing gametes without spawning, before the
2 worms start the maturing cycle once more.

3

4 The substrate housing the worms may be any
5 particulate material suitable for a deposit feeding
6 worm. Typically a sandy substrate may be used, but
7 other particulate materials (eg. glass beads)
8 having particles of a similar size could also be
9 used. Sand is preferred due to its wide
10 availability and low cost.

11

12 A suitable depth of substrate is provided to house
13 the worms. A depth of approximately 5cm is
14 sufficient for the worms to form their habitual
15 housing tubes. Whilst greater depths of substrate
16 (for example up to 10cm, even 20 to 40cm) is
17 possible, this increases the associated cost of the
18 procedure. For ease of harvesting the worms the
19 minimum depth of substrate is desirable.

20

21 The sea water used in the present method may be
22 filtered seawater (eg. filtered twice through a
23 filter having 0.34 μ m pore size), a flow through
24 system receiving natural sea water or recirculated
25 in an aquaculture system incorporating
26 biofiltration, a protein skimmer and/or other water
27 treatment devices as are readily available from
28 commercial sources.

29

30 For the purposes of hygiene management, we have
31 found it convenient if the substrate housing the
32 worms contains little or no food material with no
33 additional food material being provided during the

1 time period of 14 to 24 days. The presence of
2 little or no food allows the cleanliness of the
3 water to be easily maintained to a high standard,
4 without affecting the worms adversely since the
5 time period in question is short.

6
7 The method described herein can be used to induce
8 spawning in any species of worm belonging to the
9 family Arenicolidae. Species of particular
10 interest include *Arenicola marina* and *Arenicola*
11 *defodiens*.

12
13 The method is suitable for maturing females and/or
14 maturing males (as defined above) collected from
15 natural populations in the wild or, more
16 preferably, cultured according to the methodology
17 of WO-A-03/007701. Where the worms have been
18 cultured we have found that the best results are
19 obtained using worms maintained (with adequate food
20 supply) at a temperature of 16°C for 3 to 5 months.
21 Good results can also be obtained if the culture
22 temperature is 14°C or more, for a period of at
23 least one month.

24
25 For commercial purposes, it may be desirable to
26 allow male and female worms to spawn in isolation
27 in small containers of sea water and to selectively
28 mix the oocytes and spermatozoa, and to select for
29 fertilised eggs after induced spawning by the
30 methods described above.

31
32 We have found that if there are any unspawned worms
33 remaining at the end of the 14 to 24 day time

10

1 period referred to above during which the worms are
2 held at a temperature of 4 to 8°C, then these
3 unspawned worms can be induced to spawn by
4 adjusting the temperature of the sea water to 12 to
5 14°C. Generally, increasing the temperature
6 gradually is preferred and we have found that
7 progressively increasing the temperature at a rate
8 of 1°C per hour over a period of 6 to 8 hours is
9 suitable, although the exact rate of temperature
10 increase is not critical. The increase in
11 temperature can conveniently be achieved by
12 transfer of the worms to sea water (for example
13 filtered sea water or re-circulated sea water) at a
14 temperature of 4 to 8°C and wherein the ambient air
15 temperature is 12 to 14°C. For convenience the
16 worms may be placed into portable containers of sea
17 water at the appropriate temperature (4 to 6°C),
18 the container holding the sea-water and worms
19 combination being placed in a controlled
20 temperature room/incubator as appropriate. Under
21 these conditions, the temperature of the sea water
22 is gradually raised to 12 to 14°C, for example
23 13°C. Whilst it is preferable for the worms to be
24 housed individually at this stage (for example in
25 400ml of sea water), it is also possible for the
26 worms to be housed in small groups of up to 20
27 (preferably of 10 or less, more preferably of 6 or
28 less, for example 2, 3, 4 or 5) worms. Desirably
29 the worms will be housed in same-sex groups. The
30 worms housed in this way are examined at
31 approximate intervals (we have found hourly
32 examination to be suitable).

33

11

1 If female worms are observed to be spawning, the
2 eggs are obtained by placing the females in a tank
3 containing 1 to 3 litres of sea water and allowing
4 the worms to continue to spawn. After the majority
5 of the eggs have been released (as may be
6 determined by the requirement for larvae) the
7 female can be removed and rehoused. Conveniently,
8 a volume of sea water sufficient to provide a
9 concentration of 100,000 eggs per litre is added
10 prior to addition of sperm. (We generally find
11 that a volume of 2 to 4 litres sea water is
12 typically required, depending upon the fecundity of
13 the female.)
14

15 If male worms are observed to be spawning, the
16 sperm is taken into a pipette or syringe before it
17 becomes thoroughly mixed with sea water. This
18 reduces the spontaneous activation of the
19 spermatozoa. A concentrated sperm mixture obtained
20 in this way can be maintained at 5°C for up to 48
21 hours without loss of viability and used as
22 required. The sperm can be introduced into the
23 egg/sea water mixture described above to provide a
24 sperm concentration of 10^5 to 10^6 sperm per
25 millilitre. Sperm concentration can be determined
26 by use of a haematocytometer which is a microscope
27 slide with etched divisions and graduations
28 defining a known volume in the space beneath the
29 cover slip. Typically the concentration of sperm
30 will be calculated from the observation of the
31 average nuclear of sperm seen in a survey of 30
32 defined volumes. The sperm concentration could
33 also be estimated by a man of ordinary skill in the

12

1 art, by adding approximately the sperm released by
2 a male to 50ml sea water then adding 1ml of this
3 mixture to one litre of egg/sea water mixture.
4 In the event that the female worms are spawning,
5 but the male worms are not, it may be desirable to
6 induce immediate spawning of the male worms, as the
7 unfertilised eggs of the female worms have a
8 limited viability. Immediate spawning of the male
9 worms treated as described above can be achieved by
10 injection of the male worms with the fatty acid 8,
11 11, 14-eicosatrienoic acid, to give a final
12 coelomic concentration of 13 $\mu\text{g/g}$ body mass or an
13 *in vitro* concentration of $4.5 \times 10^{-5}\text{M}$ made by
14 dilution of a methanol solution with fine (eg.
15 $0.2\mu\text{m}$) filtered sea water or sterile water or
16 distilled water and injected to give a final
17 methanol concentration in the body tissues of 1%
18 v/v.
19
20 Once the sperm and the eggs have been mixed
21 together for a period of approximately 15 minutes,
22 the eggs may be counted (for example by randomised
23 sub-sampling) and transferred to suitable
24 containers (such as shallow plastic trays) at a
25 concentration of approximately 10,000 fertilised
26 eggs/litre. The larvae, once hatched, can then be
27 cultured accordingly, for example as described in
28 WO-A-03/007701.
29
30 The parent worms may be maintained at a temperature
31 of 16 to 20°C, but provided with suitable substrate
32 housing and organic materials as foodstuff.

13

1 Optionally the worms may be held at a reduced
2 temperature of 6 to 8°C for 2 to 3 days before
3 being returned to culture conditions.

4 Using the methodology described above it is
5 possible to induce sexual maturation in both male
6 and female worms of the family Arenicolidae only a
7 few months after previous spawning of these worms.
8 Such induction of sexual maturation of these
9 animals has no known precedent, the animals
10 spawning only once per annum in the wild.

11

12 Using the methodology described above it is now
13 possible to breed lugworms throughout the whole
14 year.

15

16 The present invention will now be further described
17 with reference to the following non-limiting
18 examples.

19

20 **Example 1**

21 **Induction of Sexual Maturation in the lugworm**

22 ***Arenicola marina***

23

24 Male and female *Arenicola sp.* were collected from
25 Hauxley beach, Northumberland during the summer of
26 2002. Male and female *Arenicola sp.* were also
27 collected from growth trials that had been carried
28 out at Seabait Ltd, Northumberland, United Kingdom.

29

30 Animals were introduced into concrete culture beds
31 (broodbeds) containing decomposed organic food and
32 sand as described in WO-A-03/O07701. The animals
33 were left for several months until required. At a

14

1 specified time during November/December 2002 a
2 group of approximately 50 of the animals were
3 removed and a coelomic biopsy was performed and
4 maturity status was determined. Selected animals
5 were then transferred into a small box containing
6 sand previously used in broodbeds for *Arenicola sp.*
7 and the small box placed in a controlled
8 temperature room held at $6^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After 21 days
9 at that temperature animals were removed from the
10 substrate and placed into separate pots containing
11 filtered sea water. Any waste material that was
12 depurated was removed with a pipette and discarded.
13 Once rehoused into the separate pots all animals
14 were re-sampled and given a number/code. Animals
15 were then gradually conditioned to 13°C . Sperm was
16 collected from spawning males in concentrated form
17 and stored in labelled glass vials in the
18 refrigerator at approximately 4°C . Females that
19 were spawning were removed from the small housing
20 pots and placed into individual labelled aquarium
21 tanks and the seawater made up to 2 litres using
22 filtered seawater. Each female was allowed to
23 continue spawning in the aquarium tank until the
24 batch-spawning event was deemed complete. At the
25 termination of the spawning event the female was
26 removed from the aquarium tank and returned into
27 the previously labelled pot provided with fresh sea
28 water. (The weight of the animal was recorded if
29 the animal had not commenced spawning before the
30 point of sampling.)

31

32 The water and eggs in the tank were mixed to give a
33 homogenous mixture, from which five to ten samples

1 of 0.5ml were removed and an estimate of the total
2 number of eggs determined (Table 1). All details
3 of provenance and usage were also recorded in this
4 table. Sperm, from two different males (L29♂.8 and
5 L23♂.1; Table 1), was added to the aquarium and the
6 eggs left to fertilise for 10 minutes. Volumes of
7 water from the aquarium tank containing fertilised
8 eggs were then transferred to white, shallow trays
9 and made up to 5 litres which resulted in a final
10 concentration of between 7 to 10,000 eggs per
11 litre. Trays were labelled and held at $13^{\circ}\text{C}\pm 1^{\circ}\text{C}$.
12 After 7 to 8 days the total content of the tray was
13 poured into an aquarium tank, which resulted in a
14 homogenous mixture of eggs and water. Six
15 replicate one-millilitre samples were removed from
16 the tank and larval numbers were assessed. Total
17 larval numbers and overall survival was determined
18 for each tray.

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1 **Table 1. Example of data sheet and sampling of**
 2 **eggs for spawning**

3

Female Ref.	L29♀.4	L29♀.5	L29♀.6	L29♀.7
Conditions/temp°C	Cold T/6-8	Cold T/6-8	Cold T/6-8	Cold T/6-8
Cold treatment period (days)	21	21	21	21
Initial wt (g)	3.7	3.9		
Sperm added (ml)	6	6	6	8
Fertilisation time (mins)	10	10	10	10
Count /1ml or 0.5ml	20	40	35	97
	44	46	31	112
	19	47	29	134
	29	57	29	129
	44	42	32	141
Σ	156	232	156	613
Mean	31.2	46.4	31.2	122.6
Sd	12.3	6.6	2.5	17.9
Vol. Of sample (ml)	0.5	0.5	0.5	0.5
Total volume (ml)	2000	2000	2000	2000
Water used (R/F)	R	R	R	R
Total (N)	124800	185600	124800	490400
Trays	3	4	3	10
No./tray	41600	46400	41600	49040

4

5 Larval counts are shown in Table 2.

6

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1 **Table 2. Results from larval counts**

2

Larval Counts (mean of six replicate 1 ml samples)				
Female	L29♀.4	L29♀.5	L29♀.6	L29♀.7
Date	7/1/03	7/1/03	7/1/03	7103
Tray No	7	9	5	14
1				
2	2	14	9	15
3	13	14	9	13
4		13		15
5				7
6				6
7				8
8				7
9				12
Σ	22	50	23	97
μ	7	13	8	11
Total in all trays	36667	62500	38333	53889
Total nominal survival (%)	88.1	134.7	92.1	109.9
Total larvae	110000	250000	115000	538889

3

4 **Example 2**5 **Re-initiation of maturation in the lugworm**6 ***Arenicola marina***

7

8 A sample of worms which underwent the prescribed
 9 treatment of cold and successfully produced and
 10 spawned eggs and sperm in November and December
 11 2002 as described in Example 1 were reconditioned
 12 into enriched broodbeds containing algae (as
 13 described in WO-A-03/007701) in December 2002

1 following spawning. After two months in the
2 enriched broodbeds the animals were removed from
3 the bed and placed into a pot of filtered sea water
4 and held at a temperature of 6°C for 48 hours.
5 After this cold treatment the animals were
6 gradually reconditioned into warm water conditions
7 for a further 2 months. Animals were tested
8 periodically using methods of coelomic biopsy for
9 maturity assessment.

10

11 At a late stage of maturation the animals were
12 removed from the broodbed and segregated into
13 individual pots of sea water as described in
14 Example 1. The animals were sampled and then
15 placed into cold conditioning (6°C) for 21 days.
16 The following methodologies were carried out to
17 initiate spawning and the controlled fertilisation
18 of eggs and production of larvae. Spawning was
19 successfully initiated in both males and females.
20 Results from some of the females are presented in
21 Table 3. Larval counts from the samples are
22 presented in Table 4.

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1 Table 3. Details of out-of-season spawning by
 2 *Arenicola sp.* after re-initiation of maturation via
 3 cold treatment and growth in enhanced substrates.
 4

Female Ref.	L26.¶1	L26.¶2	L26.¶3	L26.¶4
Temp. °C	6-8	6-8	6-8	6-8
Cold treatment period (days)	21	21	21	21
Sperm added (ml)	8	8	8	8
Fertilisation time (mins)	10	10	10	10
Count /1ml or 0.5ml	36	79	21	8
	25	67	19	12
	29	73	34	8
	68	92	35	5
	25	55	25	8
Σ	183	366	134	41
Mean	36.6	73.0	26.8	8.2
Sd	18.1	13.8	7.4	2.5
Vol. Of sample (ml)	0.5	0.5	0.5	0.5
Total volume (ml)	2000	2000	2000	2000
Water used (R/F)	R	R	R:F	R
Total (N)	146400	292800	107200	32800
Trays	3	6	3	1
No./tray	48800	48800	35733	32800

5
 6 R = recirculated seawater,

7 F = filtered seawater.
 8
 9
 10

20

1 **Table 4. Larval counts/survival of larvae 7 to 8**
 2 **days after fertilisation (applicable to Table 3)**

3

Larval Counts (mean of six replicate 1ml samples)				
Female	L26.♀1	L26.♀2	L26.♀3	L26.♀4
Date				
Tray No.	5	6	5	4
1				
2	7	6	2	
3	4	4	3	
4		6		
5		4		
6		5		
Σ	16	31	10	4
μ	5	5	3	4
Total in all trays	26667	25833	16333	22000
Total survival (%)	55	53	46	67
Total larvae	80000	155000	49000	22000

4

5 Larval survival was lower than those obtained
 6 during the breeding period.

7

8 Example 3

9 Using temperature manipulation to extend the period
 10 of spawning in cultured populations of *Arenicola*
 11 *marina* resulting in spawning up to 6 months later
 12 than the natural breeding season

13

14 It is possible to extend the breeding season of *A.*
 15 *marina* by manipulation of the water temperature of
 16 beds used to house the animals. The final stages
 17 of maturation leading to spawning of *A. marina* can

1 be controlled by maintaining the water temperature
2 above 13°C. Dropping the temperature below 13°C
3 initiates final maturation and consequently results
4 in spawning by both males and female *A. marina* at
5 times substantially different to the natural
6 breeding season. This substantially improves the
7 efficiency of the lugworm culture system.
8

9 Some degradation of eggs within the coelomic cavity
10 occurs when females, housed in suitable substrates,
11 are maintained at elevated temperatures
12 (temperatures above 13°C) for prolonged periods of
13 time (in excess of 2 months). There is variation
14 in egg condition within and between females. There
15 is nevertheless a significant production of
16 fertilisable eggs and or sperm outside the breeding
17 season and the embryos and larvae so produced can
18 be reared in the standard culture conditions as
19 previously described (see WO-A-03/007701).
20

21 The observed time of spawning for *Arenicola marina*,
22 in the wild in Northumberland, UK was recorded
23 between October 30, 2002 and November 4, 2002.
24

25 In excess of two hundred animals were each housed
26 in Beds L29, L28, L26, L25, L24 and L23 over the
27 summer period (May to September 2002) and
28 maintained thereafter for various periods of time
29 as described below. The water temperature provided
30 in the beds was maintained above 13°C. The change
31 in maturity status of *A. marina* in each bed was
32 monitored via sampling of worms using method of
33 coelomic biopsy as described previously. Animals

were assessed and, when deemed suitably mature (see above) the worms were removed and exposed to a cold treatment comprising exposure to 6 to 8°C for periods of up to 21 days.

Worms were removed from beds at the times presented in Table 5.

Table 5. The timing at which worms were removed from the beds and placed into cold treatment.

Month	Bed (worms removed for cold treatment)
November	L25, L29, L26
December	L23, L24
January	L28, L29
February	L24
March	Mature animals were available from L23 but larvae were not produced.
April	Mature animals were available from L23 but larvae were not produced.
May	L23

By the methods described it was possible to achieve fertilisation success in eggs derived from these worms in all months from November 2002 to May 2003 (Mature animals were present in March and April). Survival rates for larvae in May was lower than might be achieved at other times being approximately 20-30% but given the high fecundity of lugworms this nevertheless provides a means by which to obtain substantial numbers of larvae outside the natural breeding season. The standard

1 cold treatment technologies resulted in spawning
2 after the specified 14 to 21 days.

3

4 **Tables 6a-c.** provide specific examples of
5 treatments producing spawning animals and viable
6 larvae outside the normal breeding season.

7

8 The effectiveness of these treatments may be
9 further improved by keeping the larvae prior to
10 being stocked out to the production system. The
11 larvae of *A. marina* can be held in trays with sand
12 and static or recirculating seawater in excess of 6
13 months with minimum observed mortality (<20%). By
14 combining these approaches larvae can be
15 effectively stocked out to production beds
16 throughout the year.

Table 6a.

Batch 1 - Examples of females and males used for fertilisation procedures 2002/2003

Batch	Batch 1			
Date	08/11/2002	08/11/2002	08/11/2002	08/11/2002
Female Ref.	L26#14	L26#15	L26#16	L26#16
Origin	L26	L26	L26	L26
Temp.°C	6-8	6-8	6-8	6-8
Cold treatment period (days)	14	14	14	14
Initial wt(g)	6.3	3.4	4.1	4.1
Sperm added (ml)	5	5	5	5
Males	H.#1a	H.#B.4	H.#B.5	H.#B.5
	♂♂ mix H	H.#B.5	L26.#9	L26.#9
Fertilisation time (mins)	10	10	10	10
Count /1 ml or 0.5 ml	11	23	38	38
	8	24	45	45
	18	24	49	49
	28	16	44	44
	8	10	37	37
Σ	73	97	213	213
Mean	14.6	19.4	42.6	42.6
Sd	8.5	6.2	5.0	5.0
vol. of sample (ml)	1.0	1.0	0.5	0.5
total volume (ml)	5000	5000	2000	2000
water used (R/F)	R	R	R	R
Total (N)	73000	97000	170400	170400
Trays	2	2	5	5
No./tray	36500	48500	34080	34080

Table 6b.

Batches 3 and 4; Examples of females and males used for fertilisation procedures 2002/2003

Batch	Batch 3				Batch 4			
Date	07/01/2003	08/01/2003	08/01/2003	15/02/2003	16/02/2003	16/02/2003	16/02/2003	
Female Ref.	L23♀.9	L24♀.8	L24♀.10	L28♀.2	L29♀.10		L28♀.2	
Origin	L23	L24	L24					
Temp. °C	6-8	6-8	6-8	6-8	6-8	6-8	6-8	
Cold treatment period (days)	21	21	21	21	21	21	21	
Initial wt(g)	5.3	12.1	8.5	unk	unk	unk	unk	
Sperm added (ml)	6	5	5	6	4	3	3	
Males	L23♂.3	L24♂.5	L24♂.5	L28♂.9	L28♂.9	L28♂.9	L28♂.9	
	L24♂.1, 7			L28♂.3	L28♂.11	L28♂.11	L28♂.11	
Fertilisation time (mins)	10	10	10	15	15	15	15	
Count /1 ml or 0.5 ml	44	17	42	30	70	5	5	
	52	17	71	41	56	7	7	
	43	10	56	39	39	10	10	
	52	23	41	30	44	8	8	
	50	13	54	31	39	14	14	
Σ	241	80	264	171	248	44	44	
Mean	48.2	16	52.8	34.2	49.6	8.8	8.8	
Sd	4.4	4.9	12.2	5.4	13.4	3.4	3.4	
vol. of sample (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
total volume (ml)	2000	2000	2000	4300	2000	2000	2000	
water used (R/F)	R	R	R	F	F	F	F	
Total (N)	192800	64000	211200	294120	198400	35200	35200	
Trays	2	1	3	6	4	1	1	
No./tray	96400	64000	70400	49020	49600	35200	35200	

Table 6c.

Batch 6; Examples of females and males used for fertilisation procedures 2002/2003

Batch	Batch 6			
Date	13/05/2003	13/05/2003	13/05/2003	13/05/2003
Female Ref.	L23#1	L23#2	L23#9	L23#9
Origin	L23	L23	L23	L23
Temp. °C	6-8	6-8	6-8	6-8
Cold treatment period (days)	21	21	21	21
Initial wt(g)	unk	unk	unk	unk
Sperm added (ml)	7	7	7	7
Males	L23#3	L23#3	L23#3	L23#3
	Ctroom: 6°C			
Fertilisation time (mins)	20	20	20	20
Count /1 ml or 0.5 ml	192	122	165	165
	109	101	112	112
	117	111	152	152
	139	105	133	133
	171	85	141	141
Σ	728	524	703	703
Mean	145.6	104.8	140.6	140.6
Sd	35.4	13.6	20.0	20.0
vol. of sample (ml)	0.5	0.5	0.5	0.5
total volume (ml)	2000	3000	2000	2000
water used (R/F)	R	R	R	R
Total (N)	582400	628800	562400	562400
Trays	1	4	1	1
No./tray	150000	157200	150000	150000

Key: L - bed code; unk - unknown; R- recirculated, filtered sea water; F - filtered sea water

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